



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(21) International Application Number: PCT/FI91/00265 (22) International Filing Date: 27 August 1991 (27.08.91) (30) Priority data: 904214 27 August 1990 (27.08.90) FI (71) Applicant (for all designated States except US): VALTION TEKNILLINEN TUTKIMUSKESKUS [FI/FI]; c/o VTT Technology Oy, PL 402, SF-02151 Espoo (FI). (72) Inventors; and (75) Inventors/Applicants (for US only) : KANTELINEN, Anne [FI/FI]; Mateorinrata 5 C, SF-02210 Espoo (FI). PERE, Jaakko [FI/FI]; Laajaniityntie 8 D 54, SF-01620 Vantaa (FI). POUTANEN, Kaisa [FI/FI]; Lielahdentie 7 a B 19, SF-00200 Helsinki (FI). TENKANEN, Maija [FI/FI]; Kaskilaaksontie 3 C 69, SF-02360 Espoo (FI). VIIKARI, Liisa [FI/FI]; Lookkikuja 5 F, SF-00200 Helsinki (FI).		(74) Common Representative: VALTION TEKNILLINEN TUTKIMUSKESKUS; c/o VTT Technology Oy, PL 402, SF-02151 Espoo (FI). (81) Designated States: AT, AT (European patent), AU, BB, BE (European patent), BG, BR, CA, CH, CH (European patent), DE, DE (European patent), DK, DK (European patent), ES, ES (European patent), FI, FR (European patent), GB, GB (European patent), GR (European pa- tent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC, NL, NL (European patent), NO, PL, SE, SE (European patent), SU ⁺ , US. Published <i>With international search report.</i> <i>In English translation (filed in Finnish).</i>
(54) Title: A PROCESS FOR HYDROLYZING HEMICELLULOSE BY ENZYMES PRODUCED BY TRICHODERMA REESEI (57) Abstract The invention concerns a process for hydrolyzing hemicelluloses, especially xylan, using an enzyme preparation isolated from <i>Trichoderma reesei</i> , containing an endo- β -xylanase I (pI 5.5) and an endo- β -xylanase II (pI 9.0). The invention further concerns the use of this enzyme preparation in the improving of the sheet properties and the bleachability of pulps and for the production of sugars from hemicellulosic substrates.		

+ DESIGNATIONS OF "SU"

Any designation of "SU" has effect in the Russian Federation. It is not yet known whether any such designation has effect in other States of the former Soviet Union.

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A PROCESS FOR HYDROLYZING HEMICELLULOSE BY ENZYMES
PRODUCED BY TRICHODERMA REESEI

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FIELD OF THE INVENTION

The invention concerns a method for hydrolyzing hemicelluloses in wood or pulp or hemicelluloses isolated from these using enzyme preparations which are composed essentially of one or two characterized xylanases produced by Trichoderma reesei.

BACKGROUND OF THE INVENTION

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Depending on the species, wood contains about 20% of hemicelluloses, of which xylans form an essential part both in hardwoods and softwoods. Hardwoods contain 15-30% glucuronoxylan and softwoods 7-10% arabinoglucuronoxylan. During pulping processes, part of the hemicellulose is dissolved, but part remains within the fibres. The structure of the hemicelluloses remaining in the fibres affects their physical and functional properties. Depending on the type of pulping method used, soluble hemicellulose fractions differing with respect to their chemical composition and molecular weight distribution can be obtained. Even some dissolved hemicelluloses may have a high molecular weight, ie. these hemicelluloses are composed of polymeric sugar chains (xylans). Hemicellulose fractions can also be isolated from wood using methods especially developed for this purpose, e.g. for the production of xylose.

Depending on the composition of hemicellulose, several enzymes are needed for the hydrolysis. Of primary importance in many applications are the endo- β -xylanases (EC 3.2.1.8.), which hydrolyze the xylan backbone chains. Xylanases can be produced by several different micro-organisms. In an enzyme production process, these organisms usually produce several different hemicellulose-degrading enzymes into the culture broth. These contain enzymes hydrolyzing the backbone chain as well as enzymes capable of splitting off side chains or side groups attached to the backbone (endo-xylanases, β -xylosidase, arabinosidase, α -galactosidase, glucuronidase,

acetyl xylan esterase etc). In addition, within a certain type of enzyme group, individual enzymes hydrolyzing the same substrate using a different mechanism can be isolated. Examples of such enzymes are e.g. the well documented cellulases (cellobiohydrolases I and II, endoglucanases I and II etc). These enzymes differ from each other with respect to both their biochemical structure and their mode of action.

Trichoderma reesei is frequently used for the production of both cellulases and hemicellulases. The ability of this organism to produce different enzymes has been widely described, as well as the methods for separation and purification of these enzymes, especially cellulases. By contrast, the biochemical properties of different xylanases produced by this organism and moreover, the exploitation of these specific properties for different modifications of wood-derived hemicelluloses is not well known.

Trichoderma reesei produces several xylan-degrading enzymes, of which, however, only two are specific for xylan (Biely and Markovic, 1988). The other known xylanases also degrade cellulose, which in pulp and paper applications often is a detrimental property. Different species of the fungus Trichoderma have been used for production of xylanases. Generally, one main xylanase has an isoelectric point (pI-value) above 8 (e.g. Gibson and McCleary, 1987, Dekker, 1985, Wong et al. 1986). In only one study has another xylanase, with an isoelectric point of 5.1, been purified and characterized (John and Smith, 1988), however, from a strain of Trichoderma lignorum.

In this invention, two functionally different xylanases and their applications are described. The invention is based on the unexpected observation that two xylanases produced by Trichoderma reesei possess essentially different properties which can be exploited in different practical applications.

In this invention the two xylanases were separated from each other using as such known protein purification methods. Anionic and cationic ion exchangers were used for separation of the proteins. The methods used were unexpectedly rapid and simple. This invention, however, is not limited to this protein purification method, but the desired proteins can also be purified by other methods. The desired proteins can also be produced using

Trichoderma reesei strains which have been genetically modified to produce large amounts of one or both of these proteins or with other genetically modified production organisms, to which genes encoding one or both of these T. reesei enzymes have been transferred.

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The essential observation of this invention is the possibility to exploit the functional differences between these two xylanases in practical application processes. Xylanases can be characterized according to their optimal activities in different conditions or to their abilities to hydrolyze different substrates. In addition, enzymes may show different hydrolysis patterns for the same substrate. The enzymes described in this invention differ surprisingly from each other with respect both to their pH-optima and to their xylan-solubilizing and saccharifying activities against different substrates. In this patent application, the enzymes are denominated following the international enzyme nomenclature, according to which the enzymes are numbered in the order of increasing isoelectric point (pI-value). Thus, the xylanase enzyme having the lower pI-value (pI 5.5) is named as xylanase I and the xylanase enzyme having the higher pI-value (pI 9.0) is named as xylanase II. It is characteristic of the enzymes described in this invention that one enzyme (xylanase I) has optimal activity in the more acid pH region, whereas the other (xylanase II) has its pH-optimum in the near-neutral pH range. Xylanase I typically solubilizes xylan more efficiently, whereas xylanase II produces reducing sugar units more efficiently. Xylanases I and II also differ from each other with respect to their ability to degrade chemically modified xylans with a low degree of substituents (side groups). Xylanase I is relatively more efficient in the hydrolysis of this type of modified substrate. These properties can advantageously be utilized in different applications, i.e. the most suitable enzyme can be chosen according to the conditions (pH) and the structural properties of the substrate to be treated with the enzymes. Depending on the process and the wood species used in the process, the chemical structure of the substrate (xylan) varies especially with respect to the amount and type of substituents on the xylan backbone. These structural differences are widely described in the literature of the field.

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Several processes and methods based on the utilization of hemicellulases have been described in the literature. In these methods, the aim is to hydrolyze the hemicellulose to a greater or lesser extent. These methods

include e.g. the partial hydrolysis of hemicelluloses from cellulose pulps to decrease the consumption of chlorine chemicals in bleaching or to decrease the chlorinated residues in the pulp or in the effluents (eg. Viikari et al. 1987, Clark et al. 1989, Tan et al. 1987), removal of residual hemicelluloses for the production of dissolving pulps (Paice and Jurasek, 1984, Paice et al. 1988), modification of fibre properties by a partial hydrolysis of hemicellulose (Noe et al. 1986, Fuentes and Robert, 1986, Mora et al. 1986, Pommier et al. 1989, Roberts et al. 1990) or hydrolysis of solubilized xylans to monomeric sugars. However, in the methods described, the enzymes used have consisted of undefined mixtures, and the identified specific properties of the individual enzymes have not been exploited. Neither have the enzymes been isolated from the Trichoderma reesei species. For example, in the method described by Tan et al. the cellulase-free xylanase preparation was prepared from a strain of Trichoderma harzianum. The xylanases produced were in no way characterized or separated from each other. The specific properties of individual xylanases have not been exploited in any previously published applications.

It is characteristic for the process described in this invention that, of the enzymes described here, either one or a mixture of the two may be used. The need for the enzymes depends essentially on the application being considered. A novel feature of the invented method is that of the two xylanases described the most favourable combination can be designed for each application. For example, when a high degree of hydrolysis of xylan is desired, according to the method invented, it is advantageous to prepare an enzyme mixture containing both the solubilizing activity and the saccharifying activity. When the substrate contains mainly soluble, small molecular weight oligosaccharides, it is advantageous to utilize mainly xylanase II. On the other hand, when only a partial hydrolysis of chemically modified, fibre-bound xylan is desired (as in the pretreatment for bleaching), it is advantageous to use only xylanase I or a mixture containing it. If the aim is to improve fibre properties by a partial hydrolysis of the xylan within the fibres (as also for reduced energy consumption in the production of mechanical pulps), it is more advantageous to use only xylanase II or a mixture containing it. As a basis for choosing the most advantageous enzyme, their different pH-optima can also be exploited; xylanase I in the pH range of 3-6 and xylanase II in the pH

range of 4-7. When a mixture of both is used, the pH can most advantageously be 3-7.

In the following the invention will be examined in more detail with the aid of non-limiting working examples. The isolation and characterization of the enzymes are described in examples 1, 2 and 3 and their applications in examples 4,5,6 and 7.

The xylanase activities of the enzymes are determined using two methods: the XYL-DNS method measuring the formation of reducing sugars from xylan, as described by Poutanen and Puls (1988), and the XYL-SOL method which measures the xylan solubilizing activity, as described by Bailey and Poutanen (1989). The saccharifying activity is the more common method used for determining xylanase activity both in the scientific literature and in the characterization of commercial xylanase preparations, although many variations of the method exist. Cellulase activity is analyzed as activity degrading hydroxyethylcellulose (IUPAC, 1987).

When comparing different enzymes, they can be dosed either on the basis of enzyme protein or activity units. The former method reflects the functional differences (specific activity), whereas the latter is more practical when comparing e.g. different commercial preparations.

EXAMPLES**Example 1. Purification of the enzymes.**

- 5 Purification of the enzymes was started by chromatography using a cation exchanger (CM-Sephadex) at pH 4, adding sodium chloride to develop a linear concentration gradient between 0 and 0.15 M. The desired enzymes were collected into these fractions. The first xylanase (xylanase I) with a pI value later determined to be 5.5, was further chromatographically purified on an anion exchanger (DEAE-Sephadex) at pH 7.0. The
10 second xylanase (xylanase II), pI value 9.0, was further purified on a cation exchanger (CM-Sephadex) at pH 8.0.

Example 2. Characterization of the enzymes.

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The protein properties of the enzymes purified according to example 1 were further characterized by standard methods used in protein chemistry. These properties are described in Table 1.

20 Table 1. Properties of the Trichoderma reesei xylanases.

Enzyme	Isoelectric point, pI	Molecular weight (kDa)	pH-optimum
25 Xylanase I (Xyl I)	5.5	19	4.0-4.5
Xylanase II (Xyl II)	9.0	20	5.0-5.5

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Example 3. Substrate specificities of the enzymes.

The properties of the enzymes purified and characterized according to examples 1 and 2 were further described according to their abilities to hydrolyze different substrates. The results are summarized in Table 2.

Table 2. Specific activities of the enzymes.

Enzyme	Specific activity		
	Cellulase HEC (nkat/mg)	Xylanase XYL/SOL (U/mg)	Xylanase XYL/DNS (nkat/mg)
Xylanase I	0	630	1700
Xylanase II	0	430	7000

Example. 4. Hydrolysis of isolated hemicellulose.

Xylan isolated from a waste liquor originating from hardwood was hydrolyzed using xylanases I and II isolated according to example 1. The amount of enzyme used was 150 $\mu\text{g/g}$ of substrate dry weight when only one xylanase was used. When the two xylanases were applied, each of the enzymes was dosed at half of this amount. The hydrolysis experiments were carried out at 45 °C, at pH 5 and the hydrolysis time was 24 hours. The hydrolysis results, corresponding to the properties of the enzymes, are presented in Table 3. According to the results, it is obvious that when a high degree of hydrolysis is required, it is most advantageous to use a mixture of both enzymes. For a high degree of hydrolysis, both high solubilizing and saccharifying activities are needed in the enzyme preparation.

Table 3. Hydrolysis of waste liquor xylan.

5	Enzyme	Hydrolysis result*
		(% of original substrate)
10	XYL I	26
	XYL II	33
	XYL I + XYL II	40

* In the hydrolysis result, the total amounts of small molecular weight hydrolysis products; xylose, xylobiose, xylotriose and xylotetraose were included.

15 Example. 5. Bleaching of kraft pulp.

Unbleached pine sulphate pulp (kappa number 34,1) was treated at a consistency of 5 % with the T. reesei xylanases XYL I and XYL II at 45 °C for 4 hours. The original pH value was adjusted to the optimum of each enzyme. The enzymes were dosed at 2 or 10 µg protein/g dry pulp. The reducing sugars released in the enzymatic treatment are presented in Table 4. In this partial, incomplete hydrolysis of xylan, xylanase II was able to liberate somewhat more reducing sugars than xylanase I during the four hours' hydrolysis test, as expected when considering the better ability of xylanase II to liberate reducing sugars. Oligomeric compounds detected in the solubilized fraction from the pulp were formed slightly more after the treatment with the xylanase I.

In Table 4 the activity units, expressed both as solubilizing and saccharifying activity units, corresponding to the enzyme protein amounts used, are also presented. As the saccharifying activity is the most commonly used unit for describing the activity of an enzyme preparation, it is most convenient to use it also as a basis for comparison of individual enzymes.

Table 4. The amount of sugars liberated from unbleached pine kraft pulps after treatments with XYL I and XYL II (4 hours' treatment, enzyme dosage 2 $\mu\text{g/g}$ and 10 $\mu\text{g/g}$ pulp).

5	Enzyme PROT. (μg/g)	XYL/SOL (U/g)	XYL/DNS (nkat/g)	Red. sugars (g/g pulp)
	XYL I 2	1.2	3.4	0.20
	XYL I 10	6.3	17	0.40
10	XYL II 2	0.9	14	0.24
	XYL II 10	4.3	70	0.54

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After the enzymatic treatments, the pulps were chemically bleached using a chlorine bleaching sequence, where in the prebleaching stage the amounts of chlorine gas and chlorine dioxide were the same (calculated as active chlorine). The entire bleaching sequence was: (D50/C50)EDED. In the reference bleaching the chlorination factor was 0.18. In the enzymatically treated pulp, the amount of active chlorine was decreased by about 20 %, resulting in a chlorination factor of 0.15. After bleaching, the brightness-values, viscosities and intermediate kappa numbers (describing the lignin content of pulp after the prebleaching stage) were determined. The bleaching results are presented in Table 5.

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Table 5. Bleaching of pine kraft pulps using purified xylanases XYL I and XYL II in a bleaching sequence of (D50/C50)EDED. Original kappanumber 34.1.

30	Enzyme/dosage	Chlorination factor	Kappa after prebl.	Brightness (%)	Viscosity (dm ³ /kg)	
	XYL I	2 µg/g	0.15	5.3	90.4	1080
		10 "	0.15	5.1	90.8	1070
35	XYL II	2 µg/g	0.15	5.3	90.4	1080
		10 "	0.15	5.0	90.8	1070
	Reference		0.18	4.7	90.0	1050
40	Reference		0.15	6.6	89.0	1070

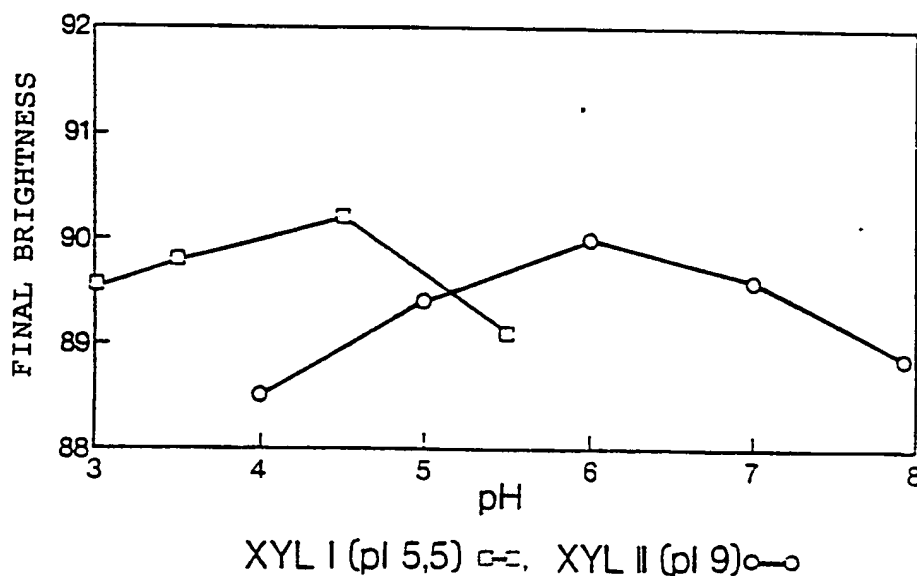
It is obvious from the results that both the xylanases, when dosed as protein were able to increase the bleachability of kraft pulp equally efficiently. When, however, the xylanases were dosed on the basis of XYL/DNS activity (according to Table 2) it can be concluded that xylanase I acts more efficiently in the bleaching. Using the same dosage of the saccharifying
 5 XYL/DNS activity, 14-17 nkat/g of substrate (equivalent to 10 μ g of xylanase I and 2 μ g of xylanase II), a better bleaching result can be obtained with xylanase I.

Example 6. Bleaching of kraft pulp.

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Unbleached kraft pulp was enzymatically treated at a consistency of 2.5 % according to example 5 with xylanases I and II at pH-values of 3-7, at 45 °C for two hours. The enzymes were dosed at 100 nkat/g (XYL/DNS). After the enzymatic treatments the pulps were bleached according to example 5 and the final brightness values were determined. The
 15 results are presented in Figure 1. It can be seen that xylanase I is more efficient in the acid pH region, whereas xylanase II acts better in the neutral pH range.

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Figure 1. Bleaching of kraft pine pulp using xylanases I and II at different pH-values.

Example 7. Improvement of fibre properties of mechanical pulp.

Coarsly refined spruce mechanical pulp (TMP, freeness 450) was treated with purified xylanases I and II at their pH-optima at 45°C for two hours. The enzyme dosage was 500 nkat/g of pulp (XYL/DNS). After the enzymatic treatments the pulps were refined in a PFI-refiner to a freeness value of about 100. The parameters describing the sheet properties were determined. The results are presented in Table 6. The percentage values in parentheses describe the extent of the positive (> 100%) or negative (< 100%) effect.

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Table 6. The properties of mechanical pulps treated with xylanases.

15	Treatment	Tensile index (Nm/g)	Tear index (mNm ² /g)	Zero-span Tensile index (Nm/g)
	Reference	25.6	5.04	81.3
	XYL I	23.0 (90%)	4.27 (85%)	78.6 (97%)
20	XYL II	26.5 (104%)	5.24 (104%)	85.5 (105%)

As is obvious from the results, xylanase II had a clear positive effect on the paper technical properties of the TMP-pulp. The xylan in mechanical pulp resembles native xylan, with a negligible loss of side chains.

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CLAIMS

1. A process for hydrolyzing hemicelluloses, especially xylans, in wood or in fibres, or isolated from these, characterized by utilization of an enzyme preparation isolated from Trichoderma reesei, containing an endo- β -xylanase with a pI value of 5.5 and a molecular weight of 19 kDa and/or an endo- β -xylanase II with a pI value of 9.0 and a molecular weight of 20 kDa.
2. The process according to claim 1, characterized by using an enzyme preparation produced by the fungus Trichoderma reesei, by a strain derived from that or by any other host strain, to which the genes encoding T. reesei xylanases I and/or II have been transferred.
3. The process according to claims 1 or 2, characterized by using an enzyme or a mixture of enzymes chosen according to their pH optima and the nature of the application.
4. The process according to claims 1, 2 or 3, characterized by using xylanase I in the pH range 3-6, preferably 4-5, and xylanase II in the pH range 4-8, preferably 5-7 or a mixture of these, when the pH is around 3-8, preferably 4-7.
5. The process according to claims 1, 2, 3 or 4, characterized by using xylanase I or xylanase II, or a mixture of them, preferably xylanase II, for improving the sheet properties of pulps.
6. The process according to claims 1, 2, 3 or 4, characterized by using xylanase I or xylanase II, or mixture of them, preferably xylanase I, in pretreatment for improving the bleachability of cellulose pulps.
7. The process according to claims 1, 2, 3 or 4, characterized by using xylanase I or xylanase II, preferably a mixture of these, for the production of sugars from hemicellulosic substrates.

INTERNATIONAL SEARCH REPORT

International Application No PCT/FI 91/00265

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC5: C 12 N 9/42, D 21 C 9/10 // C 12 S 3/08		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
IPC5	C 12 N; D 21 C	
Documentation Searched other than Minimum Documentation to the extent that such Documents are Included in Fields Searched ⁸		
SE,DK,FI,NO classes as above		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	Chemical Abstracts, volume 105, no. 23, 8 December 1986, (Columbus, Ohio, US), Lappalainen, Arja: "Purification and characterization of xylanolytic enzymes from <i>Trichoderma reesei</i> . ", see page 265, abstract 205246t, & Biotechnol. Appl. Biochem. 1986, 8(5), 437- 448 --	1-7
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<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
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International Searching Authority	Signature of Authorized Officer	
SWEDISH PATENT OFFICE	<i>Jonne Siosteen</i> Jonne Siosteen	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
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**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. PCT/FI 91/00265**

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on 31/10/91
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